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14. ABSTRACT

Asthma is the most common reason for hospitalization of children in military hospitals. In children with asthma, exposure to allergen results in pulmonary damage due to invasion of eosinophils. Eosinophils are inflammatory cells with limited life spans, and must be continually renewed from the bone marrow. We adapted an animal model of asthma to study the effect of allergen exposure on eosinophil progenitor cells (CFU-eo). These studies have revealed that CFU-eo are elevated in the bone marrow of asthmatic mice following allergen exposure. Cytokines such as IL-5, SCF, IL-4 and leukotrienes influence eosinophil production during onset of asthma and these cytokines originate from bone marrow stromal cells. We have determined that both stromal cells and T cells have a contributory role in both normal and accelerated eosinophil production noted in asthma. In addition, inflammatory mediators released from the lung alter stromal cell support of eosinophilopoiesis and may contribute to the chronic inflammation associate with long term asthma.

15. SUBJECT TERMS
Asthma, bone marrow, T cells, stromal cells, eosinophils

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Introduction

Asthma is the most common reason for hospitalization of children in both military and civilian hospitals[1-4]. In patients with asthma, pulmonary exposure to allergen results in bronchial hyperresponsiveness and airway inflammation mediated by eosinophils. Eosinophils are inflammatory cells, have limited life spans, and must be continually renewed from hematopoietic tissue. Exposure to allergen has also been correlated with systemic changes in hematopoietic function[5-9]. Our laboratory has demonstrated that initial exposure to allergen was associated with expansion of eosinophil progenitor cells, bone marrow eosinophilia, and accumulation of large numbers of eosinophils in both circulation and lung[10]. These bone marrow changes were regulated by a multi-step process with increased bone marrow output of eosinophils regulated by T cells. The increased CFU-eo production by athymic mice also demonstrates the critical importance of studying other regulatory mechanisms in the bone marrow, such as stromal cells. Data generated during this grant indicate that stromal cells may contribute to increased eosinophil production found during asthma. Our preliminary data generated during project years 01 through the 04 (addendum) also suggest that stromal cells may also provide downregulatory signals during steady state eosinophil production and early in the development of asthma. These studies take on increased importance because little is actually known about normal regulation of hematopoiesis or the possibility that systemic inflammatory responses may alter these mechanisms.

Body

Original Aims.

Research Objective 1: To determine cellular mechanisms that regulate bone marrow eosinophilia following allergen challenge. In our initial attempt to dissect regulation of eosinophil development in the bone marrow, we found that bone marrow stromal cells produce IL-5 and supported eosinophil differentiation in vitro. IL-5 production by bone marrow stromal cells was upregulated by exposure to IL-1β and this correlated with increased eosinophil differentiation *in vitro*. However, other investigators have documented IL-5 production by CD3+ T lymphocytes in the bone marrow. Experiments in this specific aim will utilize T cell deficient nude mice to determine the role of bone marrow stromal cells and T lymphocytes in eosinophil progenitor cell expansion and differentiation that lead to bone marrow eosinophilia.

Research Objective 2. To determine the effect of inflammatory mediators associated with asthma on stromal cell function. Previous experiments from this laboratory revealed that exposure of stromal cells to IL-1 and IL-4 resulted in failure of their ability to support early events in B lymphocyte development. In this specific aim we will determine the effect of inflammatory mediators that are systemically elevated in asthma on stromal cell cytokine production and function. Specifically, we will investigate stromal cell support of myeloid and lymphoid progenitor expansion. *Due to mandated decrease in the final award, the second research objective was removed in post-award budget negotiation.*

Research Objective 3: To determine the kinetics of altered bone marrow cell function in asthma. The duration of altered hematopoietic cell production following pulmonary allergen exposure is not known. This question is pertinent to the sensitization and subsequent development of childhood asthma. Establishing the kinetics of this response will be particularly important in understanding whether the bone marrow response changes with repeated exposure to allergen. Experiments in this

specific aim are designed to determine the durability of altered hematopoiesis following single or repeated pulmonary exposure to allergen.

Statement Of Work (Revised 12/31/01)

Project Year 01: In the first year of this project, we will initiate the *in vitro* and in *vivo* studies described in Research Objective 1. Although our laboratory is experienced in rodent surgery and we have an attending veterinarian consulting on this aspect of the project, it is expected that development and conduct of the diffusion chamber experiments will require a total of 30 months and will extend through the second year of the project and be concluded in Project Year 03. Completed studies will be presented at appropriate scientific meetings and prepared for publication in refereed journals.

Project Year 02: In vitro studies initiated in Project Year 01 (*Research Objective 1*) will continue throughout Project Year 02. We will initiate studies proposed in Research Objective 3 that focus on the durability of effects of repeated *in vivo* allergen dosing regimens on bone marrow function. Completed studies will be presented at appropriate scientific meetings and prepared for publication in refereed journals.

Project Year 03: During Project Year 03, we will complete remaining in vivo diffusion chamber studies described in Research Objective 1. We will complete studies of long-term allergen exposure and evaluate bone marrow transplantation studies proposed in Research Objective 3. We will repeat studies in each Research Objective 1 and Research Objective 3 as necessary to complete and appropriately document this project in published literature. Completed studies will be presented at appropriate scientific meetings and publications prepared for refereed journals.

Progress Report

In our statement of work, we proposed initiating studies that were to determine the cellular mechanisms, which regulate bone marrow eosinophilia following allergen challenge (Research Objective 1). These studies focused on the relative roles of bone marrow stromal cells and bone marrow T cells in regulating progenitor cell expansion and expression of eosinophilia following allergen exposure and encompassed both *in vivo* and *in vitro* approaches. In addition, we proposed to evaluate the effects of long-term allergen exposure on bone marrow eosinophilopoiesis (Research Objective 3).

Studies performed during project year one demonstrated that stromal cells functionally inhibited CFU-eo colony formation. These studies focused upon the inflammatory cytokine IL-1. IL-1 stimulated stromal cells appeared to increase production of a CFU-eo suppressive factor. Antibody inhibition studies determined that this stromal cell induced suppressive factor was IL-4. In addition, direct exposure of CFU-eo cultures to IL-4 resulted in a dose dependent suppression of CFU-eo colony formation. It was determined that T cells were not required for observed suppression of CFU-eo colony formation. We have hypothesized that inflammatory mediators released from the lung during asthma sensitization affect bone marrow hematopoietic function. We have previously demonstrated that IL-1 a systemically released inflammatory cytokine during asthma increases stromal cell production of IL-5. [11] This increase in stromal cell production of IL-5 is capable of supporting increased eosinophilopoiesis *in vitro*[11]. We now demonstrate that stromal cell production of IL-4 suppresses late eosinophilopoiesis and this suppression is enhanced by IL-1.

During project year 4 (addendum), these studies have been taken to an *in vivo* mouse model. 100 µg IL-4 was injected i.p. at 24 and 48 hours prior to establishment of CFU-eo cultures. Saline was delivered i.p. to control animals. CFU-eo cultures were established with 7.5 x 10^5 bone marrow cells/ml, and 10 ng/ml IL-5. Initial experiments indicated that IL-4 might accelerate eosinophil progenitor production. These experiments were performed during a period in which our mice were infected with hematopoietically active parvovirus, which necessitated repetition of these experiments in healthy mice to verify experimental results. Two replicates of this experiment have now been performed utilizing healthy mice and suggest that IL-4 actually decreases CFU-eo numbers *in vivo*. (See figure 1.) A third and final experimental replicate is now being planned.

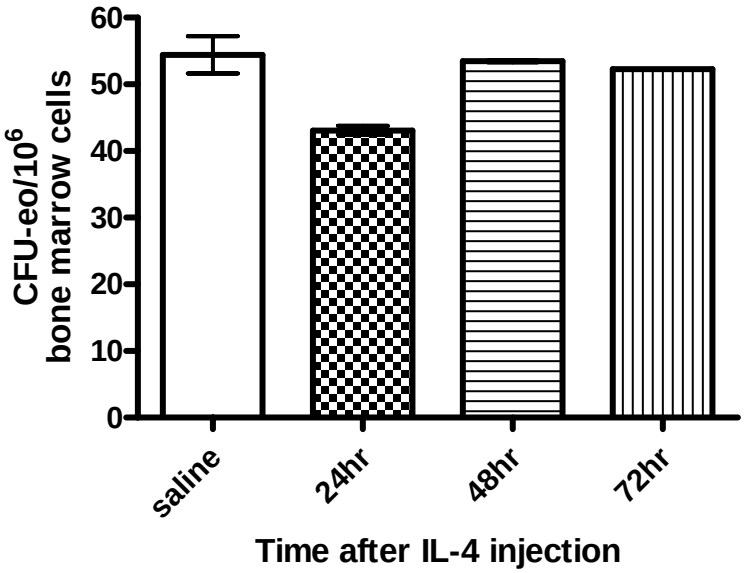

Figure 1. Effect of IL-4 in vivo on bone marrow CFU-eo.

In addition, eosinophil numbers were evaluated by applying 10^5 bone marrow cells onto cleaned glass slides 24, 48 and 72 hours after intrperitoneal injection of IL-4. Slides were stained with May-Grunwald-Giemsa stain and 200 cells counted. Mature eosinophil numbers were decreased at 24 hours after IL-4 injection and returned to normal at 48 and 72 hours after IP injection of IL-4 ($p<0.01$). This experiment has been replicated twice and a final replication is now underway.

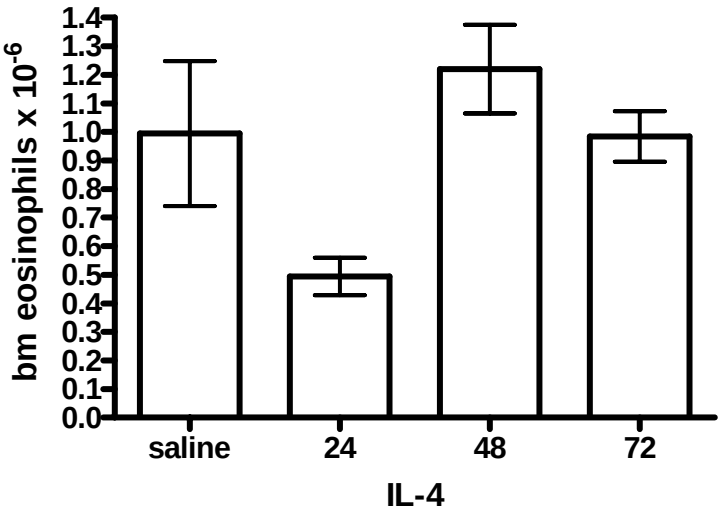

Figure 2. Effect of IL-4 in vivo on bone marrow eosinophil numbers.

We have also chosen to confirm our experimental results by utilizing an IL-4 knockout mouse model. IL-4 KO mice and control wild type mice were exposed to ovalbumin and evaluated on days 13 and 16 of the sensitization period. Bone marrow was removed and CFU-eo colonies established and counted 7 days after culture. Bone marrow eosinophil numbers were enumerated as previously described. As previously published[10], CFU-eo numbers are decreased in control wild type mice on day 13 of the sensitization period. However, in IL-4 deficient mice, CFU-eo numbers are elevated in ovalbumin sensitized mice as compared to saline controls.

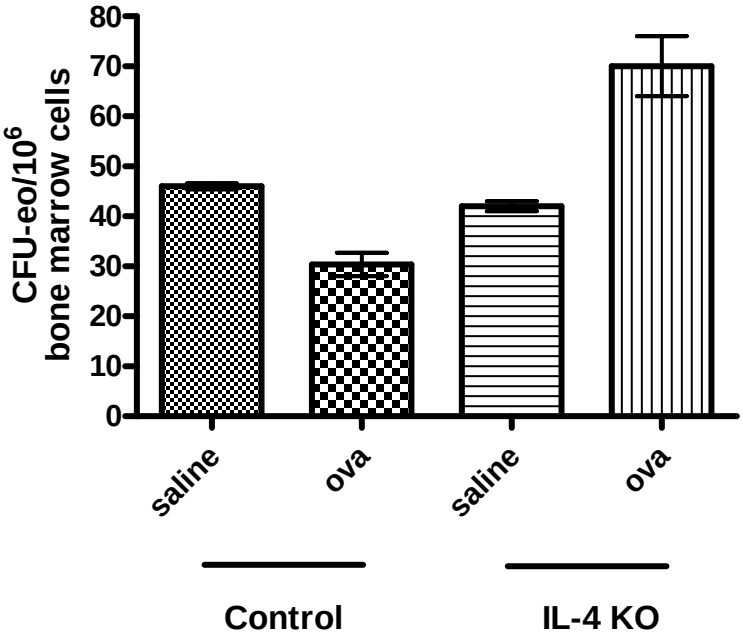

Figure 3. Effect of ovalbumin sensitization on bone marrow CFU-eo numbers in IL-4 KO mice.

It appears from these *in vivo* experiments that IL-4 has the capability of suppressing eosinophil production. These suppressive effects occur during both early and late eosinophilopoiesis. Importantly, IL-4 appears to play a significant role in suppressing CFU-eo production during the early sensitization period of asthma. In vivo studies with IL-4 KO mice are in the process of being replicated and a manuscript will be prepared when the results are verified.

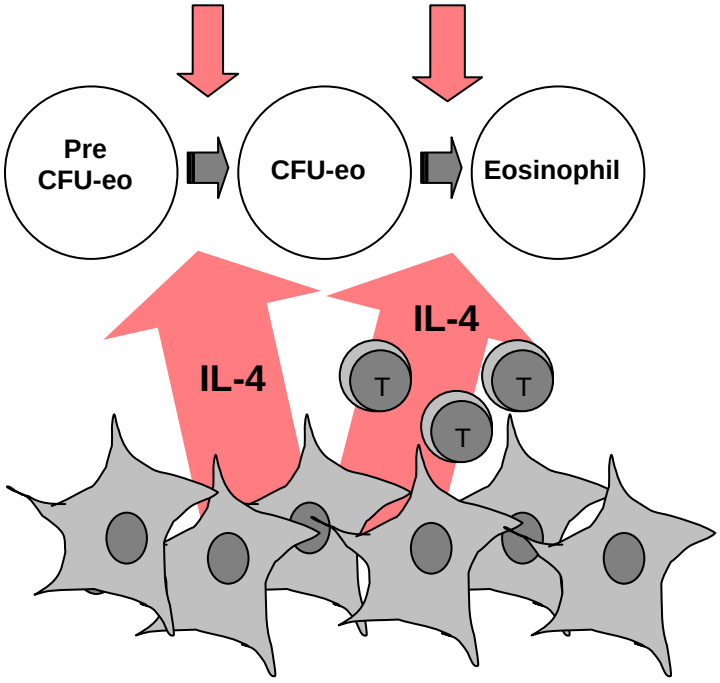

Figure 4. Effect of IL-4 on eosinophilopoiesis during onset of asthma.

Studies performed during project year one demonstrated that stromal cells functionally inhibited CFU-eo colony formation. These studies focused upon the inflammatory cytokine IL-1. IL-1 stimulated stromal cells appeared to increase production of a CFU-eo suppressive factor. When investigating potential candidate cytokines responsible for this suppress, preliminary studies performed with TGF-beta demonstrated that increasing amounts of TGF-beta in culture with bone marrow cells profoundly inhibited CFU-eo colony formation (year 1).

We have now conducted follow up studies to determine the role of TGF-β in dysregualtion of eosinophilopoiesis during onset of asthma. We have replicated our original observation by enumerating CFU-eo cells in BALB/c mice bone marrow using an assay that was supplemented with IL-5 and increasing amounts of TGF-beta. TGF β suppressed CFU-eo colony formation following addition of 0.2 ng/ml ($p<0.001$), 0.5 ng/ml ($p<0.001$), and 2 ng/ml ($p<0.001$) TGFβ.

IL-1 is an inflammatory cytokine released systemically during asthma and we have previously demonstrated that it increases bone marrow stromal cell production of IL-5.[11] In addition, this increase in IL-5 production by stromal cells resulted in increased eosinophil numbers in *in vitro* co-cultures of stroma and bone marrow. In these studies we sought to determine the source(s) of TGF-beta in the bone marrow. Bone marrow stromal cells were cultured with or without IL-1, supernatants collected

and TGF-β quantified by ELISA. Untreated bone marrow stromal cells culture supernatants contained 164 ± 11.84 pg/ml TGFβ and IL-1 stimulation decreased this amount to 104.6± 17.92 pg/ml ($p<0.02$). In addition, we determined the potential role of T cells in TGF-beta production by utilizing our T cell deficient mouse model. In these studies, BALB/c and athymic nu^-/nu^- bone marrow cells were cultured with and without IL-1(10ng/ml) and numbers of cells producing TGF-β were enumerated by Elispot. Bone marrow from BALB/c mice had 3×10^6 TGF-β cells/femur, significantly less (1.5×10^6) cells/femur producing TGF-β were found in nude mice bone marrow. No effect of IL-1 stimulation on numbers of TGF-β producing bone marrow cells was demonstrated.

CFU-eo colony formation is significantly suppressed by TGFβ. Bone marrow has several cell populations producing TGFβ; stromal cells and T cells. These data suggest that the inflammatory cytokine, IL-1, known to be released systemically following allergen exposure in asthmatic individuals, decreases bone marrow stromal cell production of TGFβ and releases a burst of accelerated production of mature eosinophils in the bone marrow. Future studies will focus on determining the exact role of TGF-beta in our murine in vivo model of allergen sensitization.

The early phase of a prototypic asthmatic response results from specific IgE activation by allergen and release of mast cell products with ensuing degranulation. Mast cells release a cascade of cytokines, which initiate the immediate phase of bronchial hyperreactivity, including leukotrienes.[12] Release of leukotrienes is directly responsible for eosinophil migration[13] and vascular permeability[14] in episodic pulmonary inflammation associated with asthma. The role of leukotrienes in observed alterations of eosinophilopoiesis has not been investigated. CysLT1 receptors are present on both hematopoietic progenitor cells and T lymphocytes in that tissue. These studies suggest that leukotrienes may be involved in both progenitor cell expansion and mature cell formation in the bone marrow. However, virtually nothing is known about the role of cysteinyl leukotrienes or their receptors in early hematopoietic production of eosinophils in the bone marrow. In these preliminary experiments the role of leukotrienes in proliferation and differentiation of eosinophil progenitor cells will be determined.

To determine the source and role of cysteinyl leukotriene production in the bone marrow during onset of asthma, we have performed studies *in vivo*. We utilized the cysteinyl leukotriene receptor antagonist; montelukast to observe the effect of decreased cysteinyl leukotrienes on eosinophilopoiesis during the onset of asthma. These studies were performed in both BALB/c and athymic nude mice to also determine the primary mechanism of cysteinyl leukotriene production (bone marrow stromal cell or T cell derived) in our murine model of asthma.

Initial experiments focused on the effects of cysteinyl leukotrienes on bone marrow eosinophilopoiesis during homeostatic conditions. In these experiments, montelukast was delivered in a biodegradable matrix subcutaneously for 16 days and then bone marrow was removed and eosinophil progenitor colonies (CFU-eo) established. On day 7 of culture, CFU-eo colonies were counted. Bone marrow eosinophil numbers were also determined. Blockade of cysteinyl leukotriene receptors resulted in a significant drop in CFU-eo in balb/c mice indicating that cysteinyl leukotrienes are partially responsible for the maintenance of eosinophil progenitor numbers in the bone marrow. (Figure 5.) When this study was also performed in athymic nude mice to determine if observed cysteinyl leukotriene effect on CFU-eo numbers was T cell or stromal cell dependent, no effect on nude mouse eosinophil progenitors was noted (data not shown). This indicates that the maintenance of CFU-eo progenitor numbers in the bone marrow under normal conditions is partially dependent upon the presence of cysteinyl leukotrienes and is mediated through T cells.

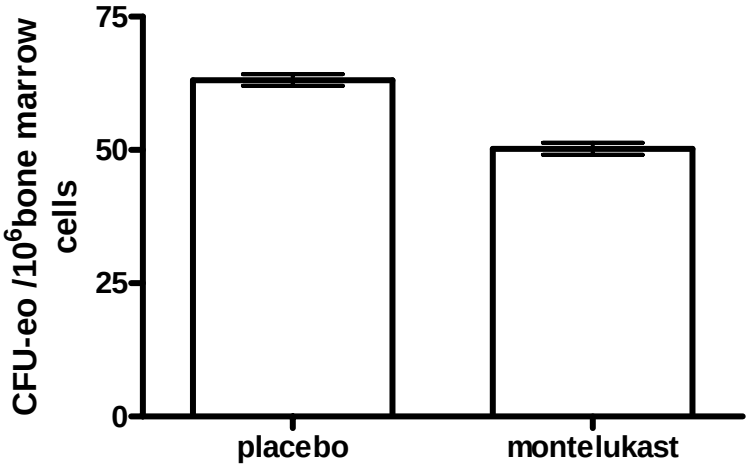

Figure 5. Effect of cysteinyl leukotrienes on bone marrow CFU-eo numbers during normal conditions.

To determine the effect of cysteinyl leukotrienes on accelerated eosinophilopoiesis noted during the early stages of asthma, montelukast or placebo was delivered in a biodegradable matrix subcutaneously for 16 days and mice simultaneously sensitized to allergen as previously described. Bone marrow was removed and eosinophil progenitor colonies (CFU-eo) established on days 13 and 16 of the sensitization period. On day 7 of culture, CFU-eo colonies were counted. Bone marrow eosinophil numbers were also determined on days 13 and 16.

Placebo exposed mice had the expected decrease in CFU-eo when sensitized to ovalbumin at day 13 (Figure 6). However, there was no associated decrease in CFU-eo numbers in montelukast treated mice indicating that cysteinyl leukotrienes are partially responsible for decreases in CFU-eo numbers during initial sensitization to allergen (Figure 6).

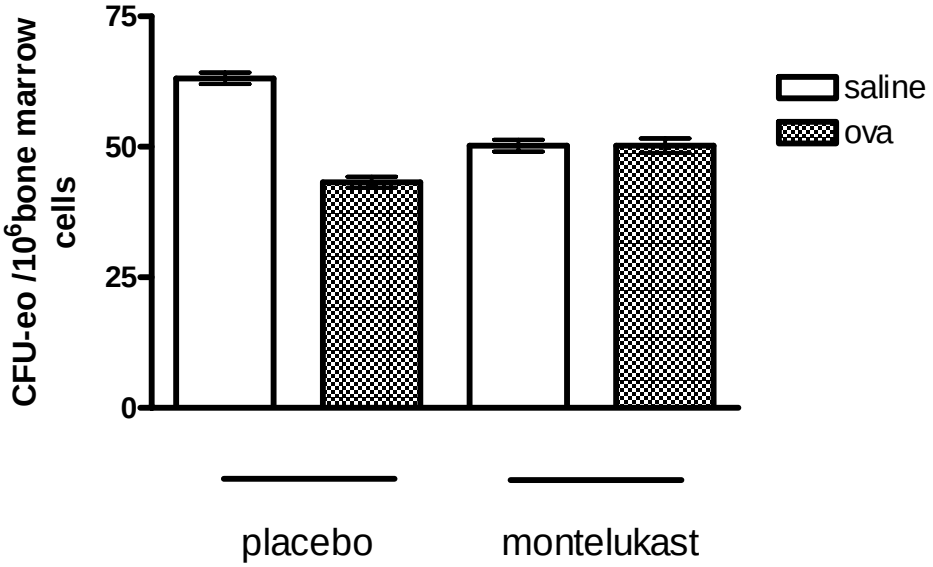

Figure 6. Effect of cysteinyl leukotrienes on bone marrow CFU-eo numbers during onset of asthma.

Studies were performed comparing mature bone marrow eosinophil numbers in saline exposed BALB/c mice to mice which received montelukast treatment. No effect of cysteinyl leukotrienes was found on mature eosinophil numbers in saline exposed mice as compared to saline exposed and montelukast treated mice. (Figure 7.) This suggests that mature eosinophil numbers in the bone marrow at steady state eosinophilopoiesis are not mediated by cysteinyl leukotrienes.

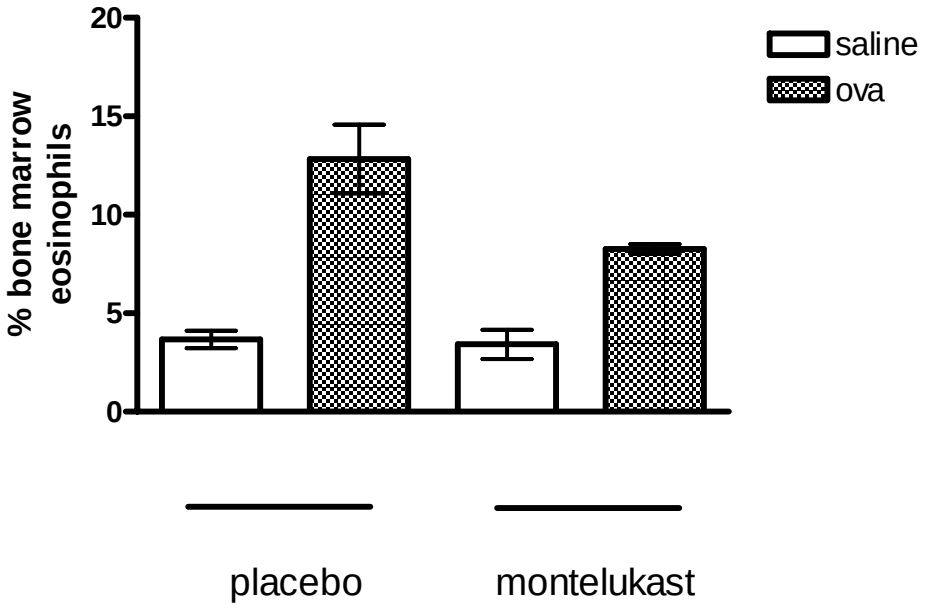

Figure 7. Effect of cysteinyl leukotrienes on bone marrow eosinophil numbers during onset of asthma.

To determine if the effect of cysteinyl leukotrienes on accelerated eosinophilopoiesis noted during the early stages of asthma was mediated through T cells, montelukast or placebo was delivered in a biodegradable matrix subcutaneously for 16 days and athymic nude mice simultaneously exposed to allergen as previously described. Bone marrow was removed and eosinophil progenitor colonies (CFU-eo) established on days 13 and 16 of the sensitization period. On day 7 of culture, CFU-eo colonies were counted. Bone marrow eosinophil numbers were also determined on days 13 and 16. In figure 7, montelukast partially blocked the rise in bone marrow eosinophil numbers seen on day 13 in ovalbumin sensitized mice as compared to placebo treated mice.

We have previously determined that the increase in CFU-eo numbers found during the early phase of asthma is T cell independent. Nude mice when stimulated with ovalbumin have a rise in CFU-eo numbers at day 13 of the sensitization phase of asthma. Blockade of cysteinyl leukotriene receptors resulted in no effect on CFU-eo numbers in T cell deficient nude mice indicating that cysteinyl leukotriene effect demonstrated previously is mediated through T cells. (Figure 8.) Previous experiments performed during year 3 of this grant suggested that the presence of T cells caused CFU-eo numbers to drop. These studies were performed in vitro and T cell transplant studies confirmed this observation *in vivo*. In the context of the finding that cysteinyl leukotrienes cause a depression at day 13 in bone marrow CFU-eo, and that this decrease in CFU-do numbers is T cell dependent, it is plausible to suggest that the mediator of T cell induced depression in CFU-eo numbers after T cell transplantation may in fact be due to cysteinyl leukotrienes. We intend to perform *in vitro* studies to investigate this possibility. In addition, we plan to replicate these studies in the next year to verify the results. A manuscript will be prepared once these studies are completed.

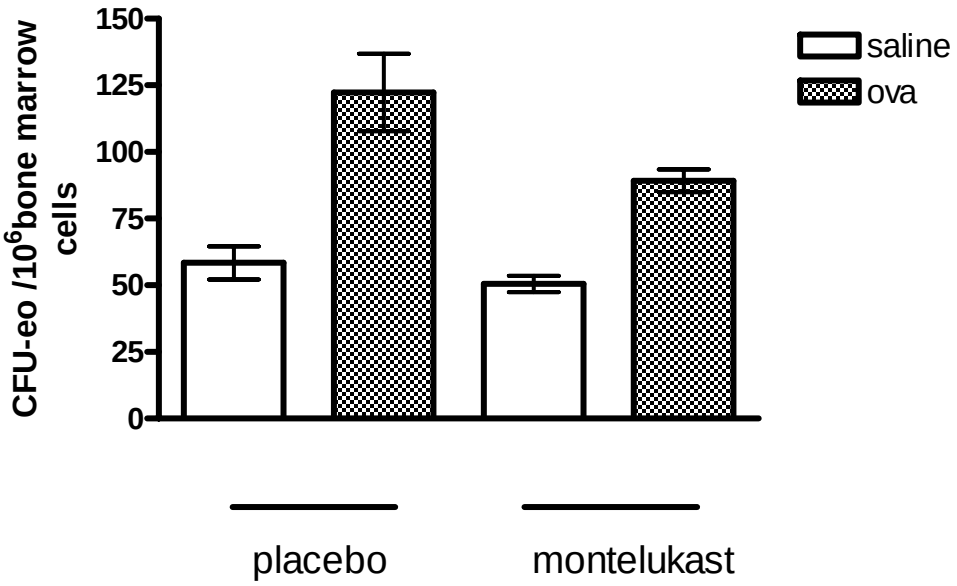

Figure 8. Effect of cysteinyl leukotrienes on bone marrow CFU-eo numbers in T cell deficient mice

We hypothesized that accelerated CFU-eo production found late in the sensitization period of asthma may result from cytokine stimulation of accelerated stem cell production in the bone marrow. Stem cell factor (SCF) is known to stimulate bone marrow stem cell proliferation.[15] Therefore, we chose to investigate the effects of SCF on early eosinophil progenitor production. In this *in vivo* study, athymic nude mice were sensitized to ovalbumin. Anti-murine SCF antibody or its isotype control antibody was administered to mice on days 9-12 of the sensitization period. These *in vivo* data suggest that SCF may be responsible for increases in CFU-eo production noted during asthma sensitization. We have also performed *in vitro* studies investigating the effect of SCF on late eosinophilopoiesis. These experiments have been replicated and reported upon in year 2 of this grant and demonstrate that SCF also accelerates late eosinophil maturation in the bone marrow. To confirm these findings we utilized a SL/SL^d, kit receptor defective mouse model to examine the role of SCF in accelerating eosinophilopoiesis *in vivo* during the onset of asthma. C-kit receptor defective mice and control wild type mice were exposed to ovalbumin and evaluated on days 13 and 16 of the sensitization period. Bone marrow was removed and CFU-eo colonies established and counted 7 days after culture. Bone marrow eosinophil numbers were enumerated as previously described.

As expected, SL/SL^d mice has significantly lower total nucleated bone marrow cells count as compared to control mice due to defective SCF signaling through the c-kit receptor (data not shown). Due to differences in total hematopoietic cell counts in c-kit receptor defective mice and their control mice, absolute CFU-eo numbers and absolute eosinophil numbers were calculated allowing direct comparison between groups of mice. In addition to lower total hematopoietic cell counts, we demonstrate that SL/SL^d mice also have lower absolute CFU-eo numbers (figure 9) and eosinophil numbers than control mice during normal hematopoiesis. During the onset of asthma, on day 13, CFU-eo numbers in control mice were significantly lower in ovalbumin sensitized mice as compared their saline controls as previously demonstrated (Figure 9). While SL/SL^d mice had significantly lower CFU-eo numbers, ovalbumin sensitized SL/SL^d mice had a depression in CFU-eo numbers as

compared to the saline exposed c kit receptor defective mice, effectively mimicking the wild-type response to ovalbumin (figure 9).

Figure 9. Effect of SCF on bone marrow CFU-eo numbers at baseline and during onset of asthma.

Studies were also performed to determine if mature eosinophil production in the bone marrow during the early sensitization phase of asthma is dependent upon SCF. SCF deficient SL/SL ^{d} mice were exposed to ovalbumin and bone marrow removed on day 13. There was no statistical difference in eosinophil numbers between ovalbumin exposed and saline exposed SCF deficient mice (Figure 10). These results suggest that SCF is partially responsible for the maintenance of eosinophil numbers in the bone marrow, and may contribute to the increase in bone marrow mature eosinophils found during the initiation of asthma (Figure 10.) We plan to verify these results by replicating the studies in the next year. A manuscript will be prepared once these studies are completed.

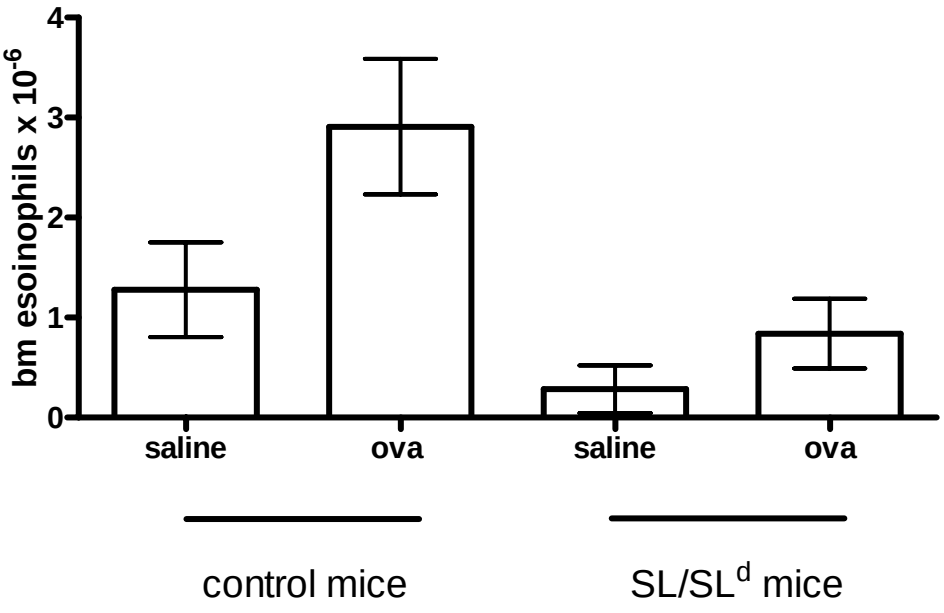

Figure 10. Effect of SCF on bone marrow eosinophil numbers at baseline and during onset of asthma.

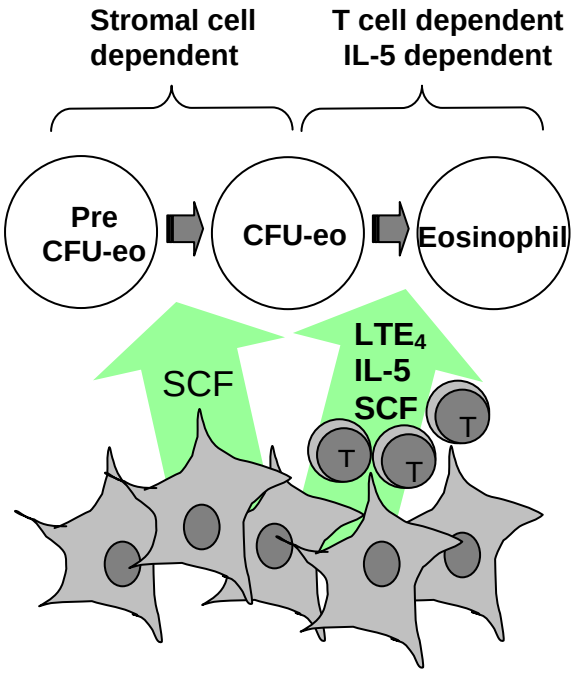

Figure 11. Inflammatory signals accelerating eosinophilopoiesis during onset of asthma.

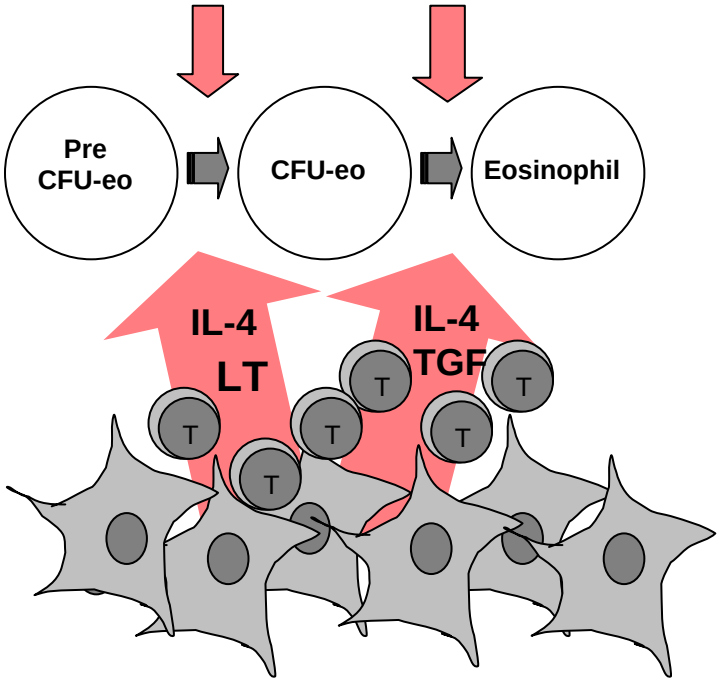

Figure 12. Inflammatory signals suppressing eosinophil production during asthma.

In research object 3, we proposed to determine the durability of the bone marrow response in asthma by establishing a repetitive challenge model more close mimicking chronic repetitive exposure to allergen in childhood asthma. In year two we initiated repetitive challenge studies as proposed in BALB/c mice. Exposure period was weekly over 2 months. In these studies, no evidence of chronic altered production of eosinophil progenitors or mature eosinophils was demonstrated. However, a publication by Shinagawa and Kojima[16] suggested that murine strain difference may be responsible for these findings. A/J mice were demonstrated in this study to have asthma features consistent with chronic asthma in humans. These findings included airway wall thickening, and persistent airway hyperreactivity[16]. The contribution of bone marrow eosinophil production to the development of these chronic asthma changes was not determined. We initially had difficulties performing these experiments due to parvovirus infection of our mice. We have recently repeated these studies utilizing Shinagawa's published methods in A/J mice, and focused on answering whether bone marrow eosinophilopoiesis is altered during chronic asthma.

Balb/c and A/J mice were exposed to IN ova or saline 3 times per week for 12 weeks. Initial studies have shown that ova exposed A/J mice developed pulmonary eosinophilia (figure 13), and goblet cell hyperplasia (figure 14) and peribronchial fibrosis (figure 15) consistent with chronic asthma.

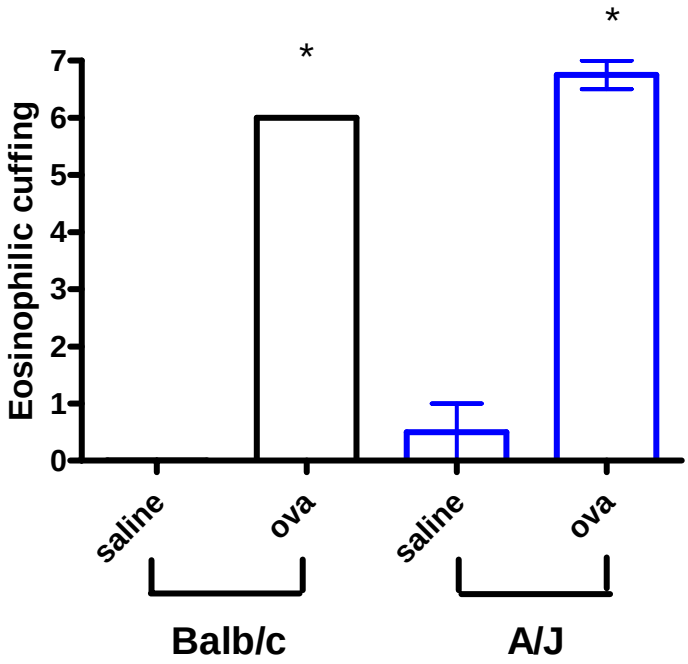

Figure 13. Eosinophilic peribronchial cuffing in chronic asthma model.

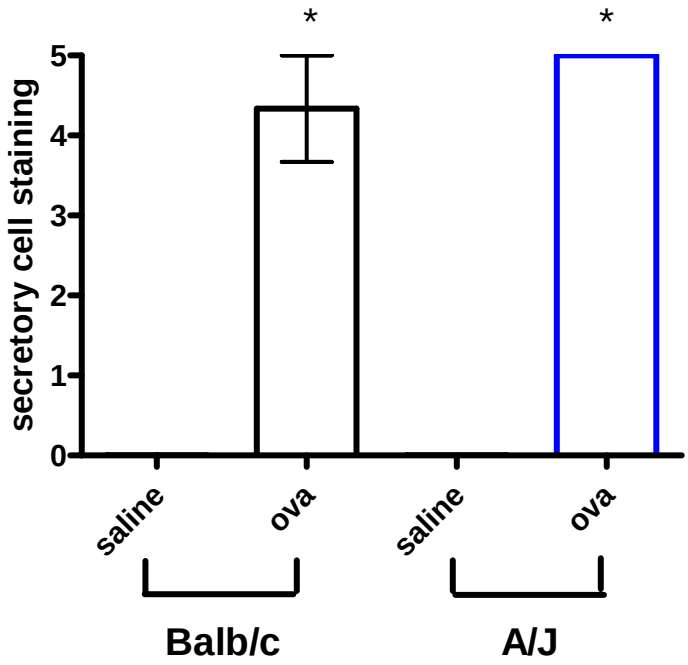

Figure 14. Pulmonary secretory cell secretion in chronic asthma model.

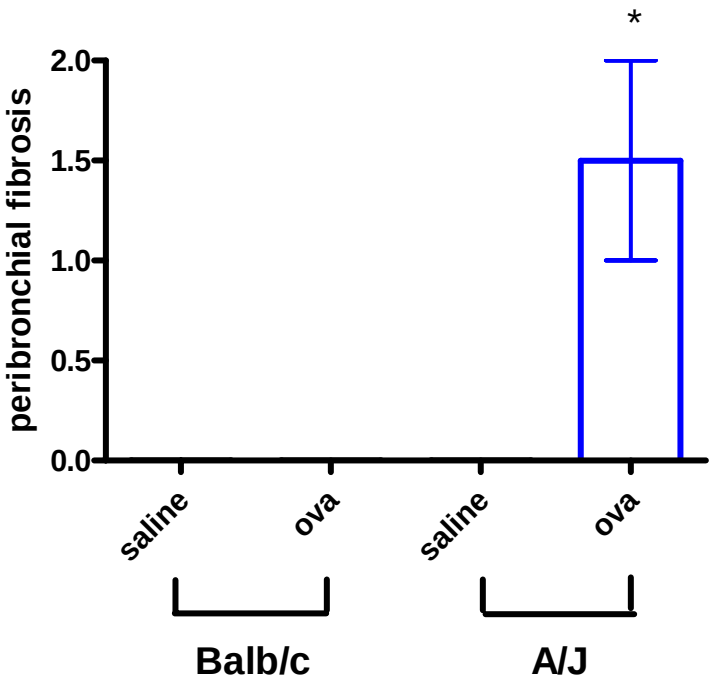

Figure 15. Peribronchial fibrosis in chronic asthma model.

Chronic pulmonary allergen exposure of BALB/c and A/J mice over the 12 week protocol did not result in altered numbers of total nucleated bone marrow cells in any of the experiments presented (data not shown). One day following the last ovalbumin dose, bone marrow CFU-eo numbers were elevated in A/J mice compared to their saline control ($p<0.05$, Figure 16). By 3 days after the last ovalbumin dose, A/J CFU-eo numbers had returned to baseline (data not shown). No differences in CFU-eo numbers in BALB/c mice were noted at any time point (data not shown). A/J mice had elevated numbers of bone marrow eosinophils at one day after last ovalbumin exposure ($p<0.05$, Figure 17). By three days after the last ovalbumin dose, A/J eosinophil numbers while still elevated ($p<0.05$) had begun to return to baseline numbers (Figure 15). Bone marrow eosinophilia was not observed in BALB/c mice in this asthma model.

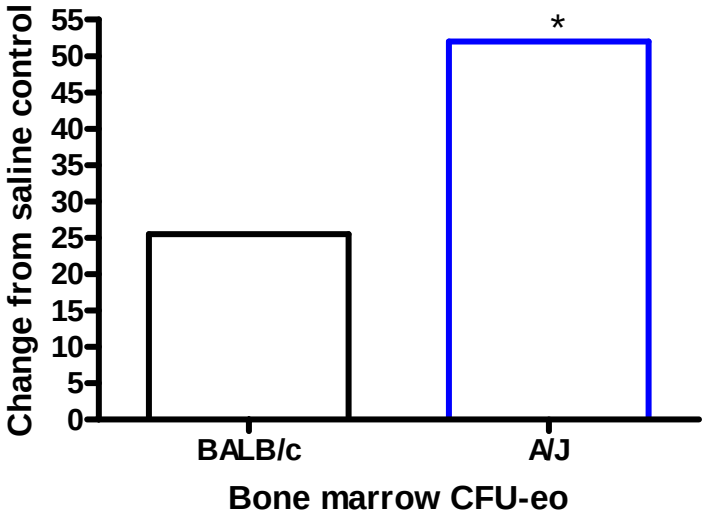

Figure 16. Effect of chronic asthma on bone marrow production of CFU-eo.

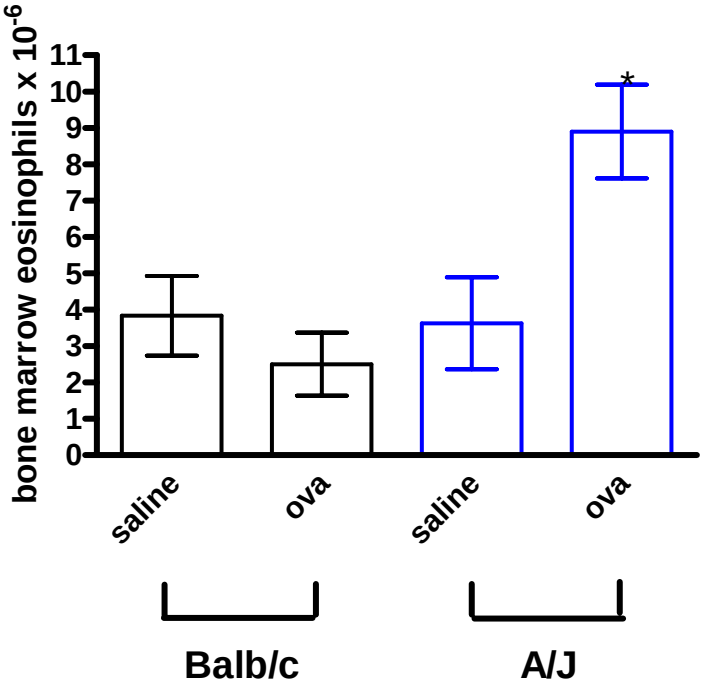

Figure 17. Effect of chronic asthma on bone marrow production of eosinophils.

Previous studies have demonstrated that eosinophils are linked to the development of airway remodeling in chronic asthma [17, 18]. In our studies utilizing two genetically different mouse strains, we observed two different bone marrow and pulmonary responses to repetitive allergen challenge. BALB/c mice while developing asthma, did not develop peribronchial fibrosis and did not have ongoing accelerated eosinophil production following allergen exposure. A/J mice continued to have ongoing accelerated bone marrow eosinophil production in response to allergen and also developed peribronchial fibrosis consistent with airway remodeling. We hypothesize that not only are eosinophils important to the development of chronic asthma and airway remodeling changes, but that the continued

responsiveness of the bone marrow to allergen may be a key factor which is associated with the development of chronic asthma.

No Cost Extension of DAMD17-02-1-0203 from 04/28/06-10/27/06

In 2003, our animal holding facilities in the Office of Laboratory Animal Resources of West Virginia University was the subject of an outbreak of *parvovirus* that proved exceedingly difficult to isolate and eradicate. *Parvovirus* specifically infects bone marrow cells and this outbreak resulted in loss of our ability to do *in vivo* studies planned as a part of our grant for a full two years while our attending veterinarian, Dr. Gary Linton, worked to resolve this persistent infection of our mice. Investigations performed in consultation with pathologists at the National Institutes of Occupational Safety and Health Respiratory Division determined that, in addition to *parvovius* infection, our mice were also suffering from a form of toxic oil syndrome linked to the new disinfectants used to eradicate *parvovirus*. In the last in the last 6-months we have finally managed to successfully repeat appropriate experiments and initiate work planned with knock-out models that are essential to our work. Since we had sufficient funds remaining in DOD Grant DAMD17-02-1-0203 to finish these experiments, we requested and were granted an additional 6 months no-cost extension of this grant end 10/27/06. this extension will allow us to publish our results, and apply for renewal of our grant. .

Key Research Accomplishments: Addendum year

- Determined that IL-4 decreases bone marrow CFU-eo numbers *in vivo*.
- Determined that IL-4 decreases bone marrow eosinophil numbers *in vivo*.
- Determined that decreased CFU-eo numbers found early in asthma are mediated through IL-4.
- Replicated that TGF-beta decreases CFU-eo colony formation.
- Identified bone marrow stromal cells as a source of TGF-beta in the bone marrow.
- Bone marrow stromal cell production of TGF-beta is decreased after stimulation with inflammatory cytokine (IL-1).
- Bone marrow T cells also produce TGF-beta.
- Numbers of bone marrow T cells producing TGF-beta are not altered by IL-1 stimulation.
- Cysteinyl leukotrienes are partially responsible for maintenance of CFU-eo numbers under normal conditions.
- Cysteinyl leukotriene effect on normal CFU-eo numbers in the bone marrow is mediated through T cells.
- Cysteinyl leukotrienes have no effect on mature bone marrow eosinophil numbers under normal conditions.
- Cysteinyl leukotrienes are partially responsible for increases in CFU-eo numbers seen during the sensitization phase of asthma.
- Cysteinyl leukotrienes are partially responsible for increases in bone marrow eosinophil numbers during the sensitization phase of asthma.
- Determined that SCF is partially responsible for maintenance of normal CFU-eo numbers during normal conditions in the bone marrow *in vivo*.
- Determined that SCF is partially responsible for maintenance of normal bone marrow eosinophil numbers *in vivo* under normal conditions.
- Determined that SCF is responsible for accelerated production of bone marrow CFU-eo numbers during the onset of asthma.

- Determined that SCF is partially responsible for elevated bone marrow eosinophil numbers during the onset of asthma.
- Replicated that chronic exposure to allergen resulted in peribronchial fibrosis in A/J mice but not BALB/c mice.
- CFU-eo progenitor cells and eosinophils were elevated in the bone marrow of A/J mice after 12 weeks of allergen exposure.
- CFU-eo progenitor cells and eosinophils were not elevated in the bone marrow of BALB/c mice after 12 weeks of allergen exposure.
- Replicated the finding that mucus containing epithelial cells were elevated in both A/J and BALB/c mice following chronic exposure to allergen.
- Replicated the finding that pulmonary eosinophilia was found in both A/J and BALB/c mice chronically exposed to allergen.
- Maintenance of bone marrow eosinophilia following chronic exposure to allergen was correlated with development of peribronchial fibrosis in mice.

Reportable Outcomes

Abstracts presented.

Ogershok P, Piktel D, Landreth KS, Hogan, MB. Effects of cysteinyl leukotrienes in regulation of eosinophilopoiesis in a murine model of asthma. Presented. AAAAI National Meeting March 2006, Miami, FL.

Hogan MB, Piktel D, Hubbs AF, McPherson L, Landreth KS. Systemic Effects of Chronic Asthma on Bone Marrow Eosinophil Development. Presented. AAAAI National Meeting March 2006, Miami, Fl.

Invited Presentations.

Cytokine regulation of early eosinophilopoiesis. Department of Pediatrics Grand Rounds. West Virginia University. July 27, 2005

Bone marrow eosinophil production in early asthma. Research presentation to the WVU Center for Respiratory Biology and Lung Disease. April 19, 2005.

Research Personnel:
Mary Beth Hogan, MD: Principal Investigator
Kenneth S. Landreth, PhD: Co-Principal Investigator
Debra A. Piktel, BS: Research Assistant III

Conclusions:

Asthma is a complex disease in which multiple mediators and cell types contribute to the pathogenesis of airway compromise. It has been recently appreciated that asthma also has systemic effects upon bone marrow regulation of hematopoiesis, in particular eosinophilopoiesis. The bone marrow environment consists of hematopoietic cells, stromal cells, mature end cells and T lymphocytes. Inflammatory mediators generated and released from pulmonary tissue, and potentially produced locally in the bone marrow during the development of asthma have the potential to exert regulatory

control on bone marrow cells. In our own studies, numbers of eosinophil progenitor cells (CFU-eo) were found to be initially depleted in the bone marrow, followed by a transient rebound to supra-normal levels. This rise in numbers of CFU-eo following allergen stimulation appeared to be regulated by stromal cells.

In the experiments presented here, we demonstrate that stromal cells from untreated mice actually secrete cytokines that inhibit eosinophil production. This inhibitory function is accelerated by exposure to IL-1 and, an inflammatory cytokine released systemically following allergen exposure in *vivo*. These data suggest that the decline of bone marrow CFU-eo that follows allergen stimulation may be due to increased suppression of eosinophilopoiesis rather than loss due to increased demand for mature eosinophils as previously reported from our laboratory. Subsequent rebound of CFU-eo and eosinophils, which has been observed following allergen exposure, is likely due to normal feedback mechanisms that regulate eosinophil homeostasis.

Our interest in childhood asthma has led us to investigate events in eosinophilopoiesis during the initial phase of the development of asthma and in events contributing to the development of chronic asthma. This investigation has led us to propose that eosinophilopoiesis is regulated in a step-wise manner by bone marrow stromal cells and T lymphocytes. Experiments to be completed during the next 6 months (no-cost extension) will be focused on replicating experiments performed during the first 3 years of the grant, but delayed due to parvovirus infection of our mouse colony. In particular, we will focus attention on the effect of cysteinyl leukotrienes on the acceleration of eosinophilopoiesis during asthma, replication of studies outlining the down regulatory signals sent by T cells to decrease CFU-eo number, and complete work on our chronic asthma model. We will also prepare these works for submission for publication once completed.

Currently there are no long-term options for intervening in the process of allergen sensitization and development of childhood asthma. Studies proposed in this grant will determine the regulatory mechanisms of bone marrow eosinophil production at both steady state and as altered in the disease state of asthma. Special emphasis on investigating the role of both bone marrow stromal cells and T cells in eosinophilopoiesis is ongoing. Preliminary data suggest that the role of T cells in eosinophilopoiesis may be complex, with T cells providing downregulatory signals early in eosinophil progenitor formation, but providing signals accelerating mature eosinophil production. In addition, ongoing studies suggest that stromal cells may provide key downregulatory signals, such as IL-4, which control eosinophil production under normal and inflammatory conditions. Stromal cells and T cells may also contribute to the accelerated eosinophilopoiesis found during asthma. Studies demonstrating that IL-5, derived from both stromal cells and T cells are responsible for accelerated mature eosinophilopoiesis. In now also is apparent that both of the cells can contribute to the increase number of immature eosinophils found during the onset of asthma in the form of SCF and cysteinyl leukotrienes. Preliminary data suggests that bone marrow eosinophilia is also a contributory factor to the development of chronic asthma. Elucidation of the normal downregulatory mechanisms of eosinophil production may lead to strategies for childhood asthma that ultimately inhibit disease development or progression.

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